# Non-Enzymatic Colorimetric Assay for
# Nitric Oxide

Catalog Number:22070
For Research Use Only

## INTRODUCTION

This kit allows you to measure total nitric oxide (NO) produced for *in vitro* experimental systems following conversion of nitrate to nitrite by metallic cadmium.
Nitric oxide can be spectrophotometrically assayed by measuring the accumulation of its stable degradation products, nitrate and nitrite. The ratio of these two products in biological fluids, tissue culture media, etc. may vary substantially. Hence, for accurate assessment of the total nitric oxide generated, one must monitor both nitrate and nitrite. An excellent solution to this problem involves the conversion of nitrate to nitrite catalyzed by cadmium. This is then followed by the quantitation of nitrite using the Griess reaction.
This kit is suitable for the quantitative determination of total nitric oxide in samples that have high protein concentrations. The cadmium catalyst proves far more robust when in the presence of harsh deproteinating reagents than the alternative, nitrate reductase.

## PRINCIPLES OF PROCEDURE

In aqueous solution, nitric oxide rapidly degrades to nitrate and nitrite. Spectrophotometric quantitation of nitrite using Griess Reagent is straightforward, but does not measure nitrate. This kit employs metallic cadmium for quantitative conversion of nitrate to nitrite (Figure 1) prior to quantitation of nitrate using Griess reagent — thus providing for accurate determination of total NO production.

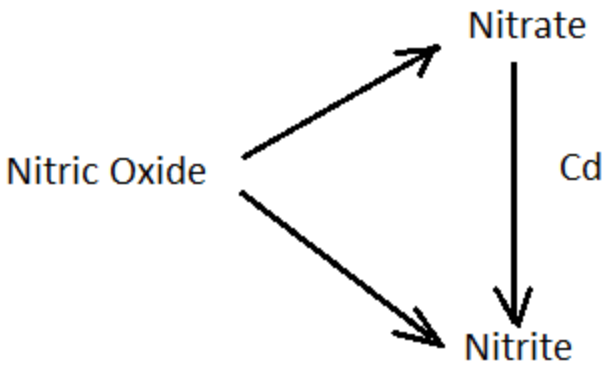



**Figure 1:** The reduction of nitrate to nitrite

This kit can be used to accurately measure as little as 1 pmol/mL (~1⌠M) NO produced in aqueous solutions. Very little sample is required (1 to 100⌠L depending on the [NO] in the sample). The completed reaction is read at 540 nm.

## MATERIALS PROVIDED

| Component | Contents | Quantity | Storage |
|---|---|---|---|
| **Cadmium Beads** | Granulated Cadmium ($Cd^{++}$) for Nitrate reduction | 25 g | 4°C |
| **$ZnSO_4$ Solution** | 30% (w/v) solution of Zinc Sulfate for deproteination | 2 mL | 4°C |

| | | | |
|---|---|---|---|
| **Microcentrifuge Tubes** | 1.5 mL microcentrifuge tubes for sample incubation | 50 tubes | 4°C |
| **Color Reagent #1** | Sulfanilamide (*p*-Aminobenzenesulfonamide) in 3N HCl | 7 mL | 4°C |
| **Color Reagent #2** | N-(1-Naphthyl) ethylenediamine dihydrochloride in $H_2O$ | 7 mL | 4°C |
| **Nitrite Standard** | 500 pmol/µL $NaNO_2$ (equivalent to 500 µM NO) | 1.5 mL | 4°C |
| **Microplate** | 96-well low-binding flat-bottom microplate | 1 plate | 4°C |
| **0.1 M HCl** | Acidic solution used to wash Cadmium Beads | 125 mL | 4°C |
| **0.1 M $NH_4OH$** | Basic solution used to wash Cadmium Beads | 125 mL | 4°C |

**MATERIALS NEEDED BUT NOT PROVIDED**

1. Adjustable pipettes (10-1,000 ⌈L) and disposable tips
2. Microcentrifuge tubes
3. Microplate reader with 540 nm filter
4. Vortex mixer
5. Deionized water

**STORAGE CONDITIONS**

1. The Cadmium Beads should be stored in a dedicated dry environment.
2. Store this kit and its components at 4°C until use.
3. Do not freeze.

**WARNINGS AND PRECAUTIONS**

1. Use aseptic technique when opening and dispensing reagents.
2. This kit is designed to work properly as provided and instructed. Additions, deletions, or substitutions to the procedure or reagents are not recommended, as they may be detrimental to the assay.
3. Exercise universal precautions during the performance or handling of this kit or any component contained therein.

**PROCEDURAL NOTES**

1. Reagents can be used immediately upon removal from refrigeration.
2. Performance of the entire kit at once is not required. When performing this kit in part, please adhere to the following:
- All unused components should be returned to their respective storage areas.
- Create a standard curve for each performance of the assay. The prepared Standards may be stored at 4°C for up to 24 hours. Sufficient stock solution of standard is provided for two standard curves.
3. To minimize error due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel.
4. Take note of the volume of each sample at the various stages of preparation and assay procedure to ensure proper dilution factor and NO concentration calculation.

**REAGENT PREPARATION**

1. **Cadmium Beads:** Cadmium Beads should be washed following the procedure below prior to use. Cadmium Beads may be prepared days prior to its intended use and should be stored in a sealed container under inert gas such as nitrogen or argon.

a. Place the desired amount of $Cd^{++}$ in an appropriately sized, sealable container. Please consider that for every 0.5 g of $Cd^{++}$ there will be 1.0 mL of wash solution applied.

b. Apply 1.0 mL of Deionized Water for every 0.5 g of $Cd^{++}$ to be used. Seal container and mix by inversion.

c. Decant the remaining solution and repeat Step b one time. Remove residual amounts of solution with a probing pipette or by dabbing with a lint-free paper towel.

d. Apply 1.0 mL of 0.1 M HCl Solution for every 0.5 g of $Cd^{++}$ to be used. Seal container and mix by inversion.

e. Decant the remaining solution and repeat Step d one time. Remove residual amounts of solution with a probing pipette or by dabbing with a lint-free paper towel.

f. Apply 1.0 mL of 0.1 M $NH_4OH$ Solution for every 0.5 g of $Cd^{++}$ to be used. Seal container and mix by inversion.
g. Decant the remaining solution and repeat Step f one time. Remove residual amounts of solution with a probing pipette or by dabbing with a lint-free paper towel.
h. The washed and dried $Cd^{++}$ is now ready for use. If you are not using the washed $Cd^{++}$ immediately, it should be stored under inert gas such as nitrogen or argon in a sealed container.

**SAMPLE PREPARATION**

This kit is designed for use with samples possessing high concentrations of protein such as serum, culture medium or tissue homogenates. Each sample should be diluted according to its presence of NO metabolites (nitrate and nitrite). Pilot studies or other research should serve as a precedent for the optimal dilution.
1. Adjust 10-50 ⌈L of sample volume to 190 ⌈L with Deionized Water.
2. Add 10 ⌈L of $ZnSO_4$ Solution to the 190 ⌈L of diluted sample, vigorously mix and incubate at room temperature (RT) for 15 minutes.
3. Centrifuge sample at 3,000 x *g* for 5 minutes.
4. Transfer the resulting supernatant to a collection tube for sample storage at –20°C or proceed to the Sample Incubation Procedure located below.

**SAMPLE INCUBATION PROCEDURE**

1. Place approximately 0.5 g of washed and dried Cadmium Beads in a dedicated centrifuge tube for each representative sample.
2. Add the deproteinated and clarified sample directly to dedicated centrifuge tube with the $Cd^{++}$ present. Incubate at RT overnight with agitation.
3. After incubation, transfer the sample to a clean microcentrifuge tube. Centrifuge for 5 minutes at 3,000 x *g*. Begin the assay within 1 hour for best results. The used Cadmium Beads should be collected and washed as indicated in the Reagent Preparation section above.

**STANDARD PREPARATION**

The stock nitrite standard is provided as a 500 ⌈M NO equivalents (500 pmol/⌈L of $NaNO_2$). Prepare the standards according to the following Table 1. The Standards do not need to be incubated with the Cadmium Beads.

**Table 1: Preparation of Standard Curve**

| Standard | NO Equivalents (µM) | Deionized Water (mL) | Transfer Volume (mL) | Transfer Source | Final Volume (mL) |
|---|---|---|---|---|---|
| $S_7$ | 100 | 2.0 | 0.5 | Standard Stock | 2.0 |
| $S_6$ | 50 | 0.5 | 0.5 | $S_7$ | 0.5 |
| $S_5$ | 25 | 0.5 | 0.5 | $S_6$ | 0.5 |
| $S_4$ | 10 | 0.75 | 0.5 | $S_5$ | 0.75 |
| $S_3$ | 5 | 0.5 | 0.5 | $S_4$ | 0.5 |
| $S_2$ | 1 | 2.0 | 0.5 | $S_3$ | 2.0 |
| $S_1$ | 0.5 | 0.5 | 0.5 | $S_2$ | 1.0 |
| $B_0$ | 0 | 0.5 | --- | --- | 0.5 |

**ASSAY PROCEDURE**

1. Add 100 ⌈L of Standards or Samples to the microplate in duplicate. **Note: S**amples may require further dilution with deionized water if the NO concentration exceeds the standard curve parameters.
2. Add 50 ⌈L Color Reagent #1 to each well and shake briefly.
3. Add 50 ⌈L Color Reagent #2 to each well. Shake for 5 minutes at room temperature.
4. Read the plate at 540 nm.

**Scheme I:**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | S7 | S7 | U1 | U1 | U9 | U9 | U17 | U17 | U25 | U25 | U33 | U33 |
| B | S6 | S6 | U2 | U2 | U10 | U10 | U18 | U18 | U26 | U26 | U34 | U34 |
| C | S5 | S5 | U3 | U3 | U11 | U11 | U19 | U19 | U27 | U27 | U35 | U35 |
| D | S4 | S4 | U4 | U4 | U12 | U12 | U20 | U20 | U28 | U28 | U36 | U36 |
| E | S3 | S3 | U5 | U5 | U13 | U13 | U21 | U21 | U29 | U29 | U37 | U37 |
| F | S2 | S2 | U6 | U6 | U14 | U14 | U22 | U22 | U30 | U30 | U38 | U38 |
| G | S1 | S1 | U7 | U7 | U15 | U15 | U23 | U23 | U31 | U31 | U39 | U39 |
| H | B0 | B0 | U8 | U8 | U16 | U16 | U24 | U24 | U32 | U32 | U40 | U40 |

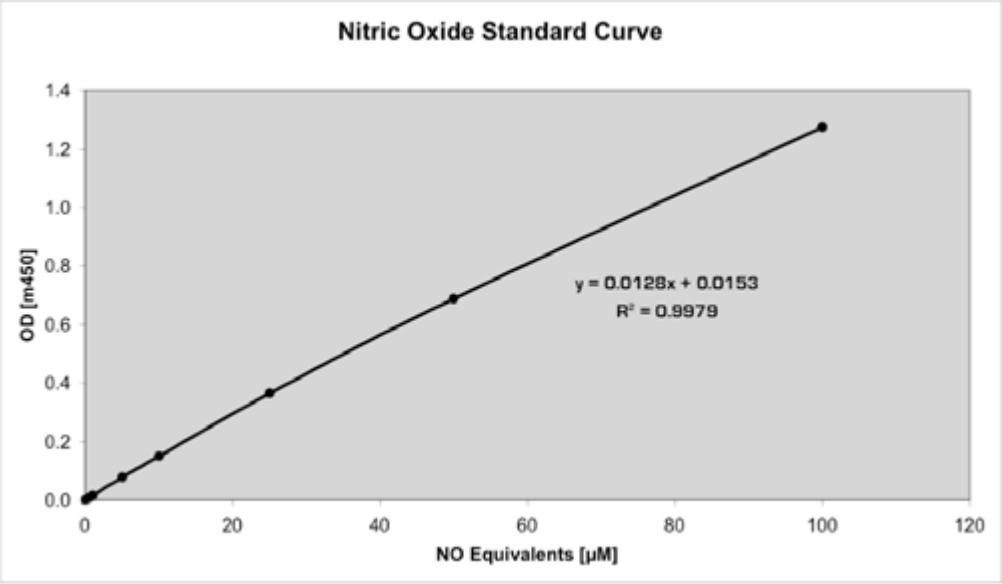
**CALCULATIONS**

1. Average the O.D. values for each replicate of sample and standard.
2. Subtract the average O.D. value of the blank wells (B0) from all other pairs of wells.
3. Plot the standard curve using the standard concentration (X-axis) vs. the corresponding O.D. (Y-axis). A typical standard curve is shown on the next page.
4. Determine the concentration of each sample by interpolation from the standard curve using the Yintercept equation. Remember to multiply by any dilution factors from the prparation steps.

**Note:** The standard curve is demonstrated in ⌈M Nitric Oxide equivalents but can alternatively be demonstrated in pmol/mL of $NaNO_2$.

1 nM NO = 1 pmol/mL $NaNO_2$

**Figure 2: Typical Standard Curve**




**REFERENCES**

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OxisResearch™

1499 Rollins Road
Burlingame, CA 94010 U.S.A.
E-mail: info@aoxre.com
Telephone: 650-289-8908
www.aoxre.com
Last revision: May 2019
Made in the U.S.A.